JC07 Rec'd PCT/PTO 30 APR 2001

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		206232US0PCT
		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) 09/830677
INTERNATIONAL APPLICATION NO. PCT/FR99/02635	INTERNATIONAL FILING DATE 28 OCTOBER 1999	PRIORITY DATE CLAIMED 30 OCTOBER 1998

TITLE OF INVENTION

USE OF AN ADSORBENT GEL FOR ELIMINATING AND PURIFYING BIOMOLECULES

APPLICANT(S) FOR DO/EO/US

Mookambeswaran YIJAYALAKSHMI, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Request for Consideration of Documents in International Search Report
Notice of Priority / PCT/IB/304 / Drawings (8 sheets) / PCT/IB/308
Article 19 Amendments (pages 16 & 17)

JC08 Rec'd PCT/PTO 30 APR 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/830677	PCT/FR99/02635	206232US0PCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1000.00
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$860.00
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$710.00
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	9 - 20 =	0	x $18.00	$0.00	
Independent claims	2 - 3 =	0	x $80.00	$0.00	
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00	

TOTAL OF ABOVE CALCULATIONS =	$990.00	
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	$0.00	
SUBTOTAL =	$990.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$0.00	
TOTAL NATIONAL FEE =	$990.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	$0.00	
TOTAL FEES ENCLOSED =	$990.00	
Amount to be: refunded	$	
charged	$	

a. ☒ A check in the amount of **$990.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER

4-30-01
DATE

DOCKET NO. 206232US0PCT

IN RE APPLICATION OF: Mookambeswaran VIJAYALAKSHMI, et al.

SERIAL NO.: New U.S. PCT Application (Based on PCT/FR99/02635)

FILED: HEREWITH

FOR: USE OF AN ADSORBENT GEL FOR ELIMINATING AND PURIFYING BIOMOLECULES

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

☒ No additional fee is required.

☐ Small entity status of this application under 37 C.F.R. §1.9 and §1.27 has been established by a verified statement previously submitted.

☐ Small entity status of this application under 37 C.F.R. §1.9 and §1.27 has been established by a verified statement submitted herewith.

☒ Additional documents filed herewith: English Translation of Specification/Notice of Priority/PCT/IB/304 Preliminary Amendment/Request for Consideration/International Search Report/PCT/IB/308 Article 19 Amendments (pages 16 & 17) /Drawings (8 sheets)/Check for $860.00

The fee has been calculated as shown below.

	(Col. 1)		(Col. 2)	(Col. 3)	SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	CLAIMS REMAINING AFTER		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE	ADDITIONAL FEE	RATE	ADDITIONAL FEE
TOTAL	* 9	MINUS	** 20	= 0	X9 =	$	X18 =	$ .00
INDEP	* 2	MINUS	*** 3	= 0	X40 =	$	X80 =	$ .00
☐	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				+135=	$	+270=	$
					TOTAL	$	TOTAL	$ .00

__ A check in the amount of $_____ is attached.

XX Please charge any additional fees for the papers being filed herewith and for which no check is enclosed herewith, or credit any overpayment to deposit Account No. 15-0030. A duplicate copy of this sheet is enclosed.

XX If these papers are not considered timely filed by the Patent and Trademark Office, then a petition is hereby made under 37 C.F.R. §1.136, and any additional fees required under 37 C.F.R. §1.136 for any necessary extension of time may be charged to deposit Account No. 15-0030. A duplicate copy of this sheet is enclosed.

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*If the entry in Column 2 is less than the entry in Column 1 write "0" in Column 3.
**If the "Highest Number Previously paid for" IN THIS SPACE is less than 20 write "20" in this space.
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206232US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

MOOKAMBESWARAN
VIJAYALAKSHMI ET AL : ATTN: APPLICATION DIVISION

SERIAL NO: NEW US PCT APPLN. :
(Based on PCT/FR99/02635)

FILED: HEREWITH :

FOR: USE OF AN ADSORBENT GEL :
FOR ELIMINATING AND
PURIFYING BIOMOLECULES

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE CLAIMS

Please amend the claims as follows:

5. (Amended) Device according to claim 2, characterized in that the biomolecule is serum β2-microglobulin.

6. (Amended) Use of the device according to claim 1 for removing biomolecules from blood, with the exception of extracorporeal dialysis.

9. (Amended) Device according to claim 1, characterized in that the device is an extracorporeal dialysis system.

REMARKS

Claims 1-9 are active in the present application. The claims are amended to remove multiple dependencies. No new matter is added. An action on the merits and allowance of the claims is solicited.

Respectfully submitted,

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206232US0PCT

Marked-Up Copy
Serial No:
Amendment Filed on:

IN THE CLAIMS

Please amend the claims as follows:

--5. (Amended) Device according to claim 2 [or claim 4], characterized in that the biomolecule is serum β2-microglobulin.

6. (Amended) Use of the device according to [claims 1 to 5] claim 1 for removing biomolecules from blood, with the exception of extracorporeal dialysis.

9. (Amended) Device according to [any one of claims 1 to 5] claim 1, characterized in that the device is an extracorporeal dialysis system.--

WO 00/25911 PCT/FR99/02635

USE OF AN ADSORBENT GEL FOR ELIMINATING AND PURIFYING BIOMOLECULES

The present invention relates to the use of an adsorbent gel combining the properties of size exclusion and affinity chromatographies (AdSEC, for "*Adsorptive Size Exclusion Chromatography*").

The principle of an AdSEC gel results from the fusion of two chromatographic techniques: size exclusion and affinity, so as to obtain supports combining the most advantageous properties thereof.

Size exclusion chromatography (gel filtration) allows the separation of molecules according to their steric bulk alone during their passive diffusion in a molecular sieve (gel). The largest molecules cannot penetrate the crosslinked matrix and are consequently excluded more rapidly from the column. This technique possesses the characteristic feature of not exhibiting interactions between the support and the molecules, and therefore of being relatively only slightly sensitive to the biochemical conditions (pH, ionic strength) of the solution. On the other hand, because of its principle of diffusion, the limiting factors for its use are generally a long operation time (because low flow rates are used), as well as a relatively limited deposition of samples (1 to 5% of the column volume).

Affinity chromatography is based on molecular interactions between the support (matrix onto which affinity ligands are grafted) and the molecules to be separated. Among these affinity ligands, immobilized metal ions, introduced in 1975 by Porath *et al.* (Nature, 1975, 21, 598-599), represent a method of separation based on the interactions (coordination bonds) between biomolecules in solution and metal ions immobilized on a support; Zn(II), Cu(II), Ni(II) and Co(II) ions are the most commonly used. This is described as immobilized metal ion affinity chromatography (IMAC).

The combined use of the principles of size exclusion and affinity chromatographies (AdSEC) has been discussed by Porath *et al.* (Int. J. of Bio-Chromatogr., 1997, 3, 9 - 17). These authors have shown that iminodiacetic derivatives of dextran bearing metal ions as affinity ligand allow size exclusion and are capable of effectively concentrating solutions by their properties of adsorption and affinity. These authors have shown that an AdSEC gel column having a volume of 5 ml could bind a high percentage of compounds having a molecular weight of between 5 kDa and 50 kDa and concentrate them about 1000 fold in a single operation.

Such supports make it possible to adsorb the smallest molecules (having affinity for the grafted ligand) at high rates and volumes (not permitted in gel filtration). Moreover, during the synthesis of the adsorbent gel, the threshold of accessibility to the affinity ligand may be modulated during the synthesis of the gel according to the size of the biomolecule to be removed or to be purified.

Terminal renal insufficiency currently affects 22,000 people in France of which 20,000 are treated by iterative hemodialysis. Only 1800 can hope to undergo transplants each year, knowing that a quarter of them will return within 5 years to hemodialysis because of a rejection while waiting for a new transplant.

The survival of the uremic individual, all methods considered, can exceed 25 years if they do not suffer from a severe cardiovascular condition. In this case, the quality of survival is profoundly impaired over the years by the osteoarticular complications of terminal uremia, at the forefront of which there are described erosive arthropathies subsequent to depositions of β2-microglobulin (β2-M).

The mechanism of onset of these arthropathies begins as soon as the renal insufficiency responsible for accumulation of β2-microglobulin appears. This protein, having a molecular weight of 11,800 Da, will accumulate in the body over the years and become

selectively deposited at the level of the cervical disks, of the shoulders, of the hips and of the wrists. Cardiac and digestive depositions have been reported. These depositions will make fragile the joint and the adjacent bone up to total destruction of the joint. Thus, a breakdown of the vertebral bodies is observed which can cause medullary compression with loss of control of the four members, irreversible articular luxations, loss of prehension in the hands and pseudofractures of the hip. Ductal nerve compressions are observed such as the carpal tunnel syndrome.

These complications irremediably lead the uremic individual toward invalidity and the bedridden state which conventional methods of dialysis cannot prevent. A transplant allows these lesions to be stabilized.

To effectively prevent these complications, it is important to be able to effectively purify the polluting components of blood, in particular β2-microglobulin, which are synthesized daily by the body and which are not, or not sufficiently, removed by the defective kidneys in dialyzed patients.

The purification of these various biomolecules can only be done on artificial membranes during dialysis, which are currently not sufficiently effective in spite of purification by filtration and nonspecific membrane adsorption.

The existing techniques for removing biomolecules, including β2-microglobulin, are currently of 3 types:

1. <u>Removal of biomolecules by hemodialysis</u>

Hemodialysis is a technique intended for subjects suffering from partial or complete renal insufficiency (Figure 1). It consists in extracorporeal treatment of blood, providing the same functions as the kidney using a membrane process. The essential part of the hemodialyzer (1) is an exchange membrane, on either side of which circulate countercurrentwise the patient's blood and the dialyzate obtained from the

hemodialysis generator (2). This technique allows the purification of the small molecular weight compounds polluting the blood, such as urea, amino acids, inorganic salts, which are normally removed by the kidney. In the case of serum β2-microglobulin, the various dialysis membranes commonly used possess two antagonistic properties:

- capture of β2-microglobulin by nonspecific adsorption on the membrane,
- generation of β2-microglobulin by detachment of this molecule which is noncovalently associated with the surface of nucleated blood cells in the major histocompatibility complex type I.

The degree of generation of β2-microglobulin is one of the criteria which define the biocompatibility of the membranes. Thus, endowed with these two antagonist properties, some membranes lead overall, during a hemodialysis session, to an increase in the concentration of β2-microglobulin, whereas others reduce it.

However, regardless of the membranes used, these results level out over periods of over one year. Thus, it has been observed that the plasma level of β2-microglobulin in uremic patients after fifteen months of dialysis was invariably increased to be between 40 and 50 mg/l (against 1 to 2 mg/l in healthy patients). Such problems of biocompatibility also exist for the other biomolecules.

2. Removal of the biomolecules by hemofiltration

Once per month, the dialyzed individual is subjected to an ultrafiltration session. The module used (1) possesses a higher cut-off than in hemodialysis (average cut-off of 40 kDa) and allows the removal, by filtration, of the small molecules from plasma, including the smallest proteins, such as β2-microglobulin (Figure 2). During an ultrafiltration session, the loss of plasma water is compensated by an equivalent supply of physiological saline (3).

The qualitative results, with respect to the removal of β2-microglobulin (purification and generation of this molecule by ultrafiltration membranes), are similar to those obtained in hemodialysis. There is thus a great influence of the nature of the membrane and of the duration of the hemofiltration. While some membranes appear to remove more β2-microglobulin over 5 hours (one session), a leveling out of the results is also observed over time. At the quantitative level, it appears that about 50% of the serum β2-microglobulin is removed per hemofiltration session. However, even if this technique is more effective for the purification of β2-microglobulin than hemodialysis, it remains inadequate for preventing and stopping the appearance of the disease. Furthermore, this technique has the disadvantage of removing numerous other small proteins apart from β2-microglobulin, since the ultrafiltrate is removed permanently.

3. Column/hemodialyzer coupling

This method has been presented as an alternative to the customary hemodialysis and ultrafiltration methods (Nakazawa *et al.*, Int. J. Artif. Organs, 1994, 17, 203-208). It consists in a serial adsorption of the biomolecules on a porous cellulose gel (350 ml of adsorbent), followed by conventional hemodialysis. In the case of β2-microglobulin, the gel is described as having a theoretical capacity for β2-microglobulin of 1 mg per ml of adsorbent. The results obtained are the best described in the literature, since in a patient in whom the initial β2-microglobulin level was 30 mg/l, this system made it possible to reduce the β2-microglobulin concentration to 10 mg/l final after 6 months of treatment. The authors presented an improvement in delaying the appearance of amyloid deposits in 2 cases out of 3, in their patients after therapy.

However, a drop in the concentration of some serum molecules (retinol binding protein, lysozymes) is

also observed after treatment. This phenomenon is attributable to the direct passage of the blood through the adsorbent, which is likely to cause problems of biocompatibility.

Thus, the existing techniques for removing β2-microglobulin and other biomolecules have mainly two limits:

- the biocompatibility of the supports, in particular for the generation of β2-microglobulin, that is to say the equilibrium between nonspecific adsorption on the membrane and the generation of β2-microglobulin during the passage of the cells in contact with them; this equilibrium determines the quantity of β2-microglobulin really removed during a hemodialysis or hemofiltration session.
- the specificity of the substrate: indeed, the techniques of hemofiltration and of aspecific binding with ligands coupled to gels lead to the undesirable removal of other molecules from serum.

A device for removing β2-microglobulin or any other biomolecule should therefore combine satisfactory (quantitative) removal with specific (qualitative) removal of the molecule in question.

In the present invention, the inventors therefore set themselves as objective:

- the use, in a device intended to remove biomolecules, of an adsorbent gel combining the properties of size exclusion and affinity chromatographies, said gel essentially consisting of a polysaccharide matrix onto which is grafted a polymer coupled to an affinity ligand (AdSEC, for "*Adsorptive Size Exclusion Chromatography*" gel) and having an adjustable cut-off of between 2 kDa and 60 kDa,
- the use of an AdSEC gel for separating and purifying biomolecules having a molecular weight of between 2 kDa and 60 kDa,
- a device intended for the removal of biomolecules having a molecular weight of between 2 kDa and 60 kDa comprising an ultrafiltration module optionally

upstream and in series with a dialysis module and using an AdSEC gel column having an adjustable cut-off of between 2 kDa and 60 kDa, said column being mounted branching off from said ultrafiltration module; this device makes it possible to dispense with the problems of biocompatibility and to specifically remove the desired biomolecules,

- a device for purifying biomolecules having a weight of between 2 kDa and 60 kDa using an AdSEC gel column having an adjustable cut-off of between 2 kDa and 60 kDa, said column optionally branching off from a filtration system; this device makes it possible to separate normal biomolecules and biomolecules modified for example by glycation.

In one advantageous embodiment, the polysaccharide matrix is agarose or is based on an agarose derivative, the polymer may be polyethylene glycol (PEG) or polypropylene glycol (PPG) and the affinity ligand may be, for example, a metal-chelating agent coupled to metal ions, a protein, a peptide, an enzyme substrate or an enzyme inhibitor.

In a preferred embodiment, the adsorbent gel consists of a matrix based on an agarose derivative onto which is grafted polyethylene glycol coupled to iminodiacetic acid (IDA) itself coupled to metal ions, for example copper(I) ions; this complex is called IMAdSEC ("*Immobilized Metal ion Adsorptive Size Exclusion Chromatography*") gel.

In an also preferred embodiment, the cut-off of the adsorbent gel is 20 kDa, thus allowing the removal or the purification of biomolecules whose molecular weight is less than 20 kDa, in particular serum β2-microglobulin.

The purification system according to the present invention possesses the characteristic feature of placing the adsorbent gel for the biomolecule to be removed branching from the circulation system for purifying. Thus, when blood is purified, there is at no time contact between the gel and the formed elements of

the blood, therefore the problems of biocompatibility (for example generation of β2-microglobulin through contact with the nucleated cells of the blood) or hemolysis of the cells in contact with the gel are avoided.

Furthermore, unlike the other techniques currently used, the purification of the biomolecule to be removed or to be purified is carried out using a ligand which will retain only this molecule. This specificity is obtained by virtue of the double sieving of the ultrafiltration membrane (which retains for example the formed elements of the blood and the large serum molecules) and of the AdSEC gel which prevents access to the ligand for other molecules with affinity for the affinity ligand but whose size is greater than the cut-off of the gel.

The other advantage of the use of this AdSEC gel is its ease of regeneration. For example, when a metal is used as affinity ligand, it may be chelated by a solution of EDTA, which makes it possible to detach any molecule adsorbed onto the gel, thus allowing cleaning of the gel, its regeneration with a new metal load and its sterilization.

The removal system according to the invention may be used for example in the context of kidney dialysis; in this case, there is an additional advantage linked to the fact that the fraction purified by passage over the AdSEC gel returns to the patient, thus limiting losses of other elements present in the blood.

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, which refers to examples as well as to the appended figures in which:

- Figure 1 represents the general diagram for renal dialysis; (1) hemodialyzer, (2) hemodialysis generator, (3) pump,

- Figure 2 represents the diagram for a hemofiltration by ultrafiltration; (1) hemofilter, (2) hemodialysis generator, (3) physiological saline, (4) pump,

- Figure 3 illustrates the chromatographies on metal ions (copper) immobilized on 3 types of gels: **A** Sépharose® 4B-IDA-copper, peak 1: nonadsorbed proteins; peak 2: elution at pH 6.0; peak 3: elution at pH 5.0; peak 4: elution at 4.0; peak 5: elution at pH 3.0; peak 6: 25 mM EDTA. **B** Novarose®- IDA-copper, peak 1: nonadsorbed proteins; peak 2: elution at pH 6.0; peak 3: elution at pH 5.0; peak 4: elution at pH 4.0; peak 5: elution at pH 3.0; peak 6: 25 mM EDTA. **C** Novarose®-PEG/IDA-copper (IMAdSEC), peak 1: nonadsorbed proteins; peak 2: elution at pH 6.0; peak 3: elution at pH 5.0; peak 4: elution at pH 4.0; peak 5: elution at pH 3.0 (1st peak); peak 5' elution at pH 3.0 (2nd peak); peak 6: 25 mM EDTA,

- Figure 4 illustrates the electrophoretic analysis of the fractions separated by chromatography illustrated in Figure 3; the numbers correspond to the fractions separated by chromatography in Figure 3; **A** Sépharose® 4B-IDA-copper. **B** Novarose®-IDA-copper. **C** Novarose®-PEG/IDA-copper (IMAdSEC). This figure illustrates the specificity of the IMAdSEC gel for β2-microglobulin relative to the two other types of gel,

- Figure 5 illustrates the analysis by mass spectrometry of the protein composition of the starting ultrafiltrate and of the fraction retained on IMAdSEC gel. **A** (I) spectrum for the ultrafiltrate, (II) deconvolution of the spectrum (a) calculation of the mass of β2-microglobulin (b) calculation of the mass of albumin. **B** (I) spectrum for the purified fraction, (II) deconvolution of the spectrum and calculation of the mass of β2-microglobulin,

- Figure 6 illustrates the capacity of the IMAdSEC gel for β2-microglobulin,
- Figure 7 illustrates the mounting, on a branch, of a filtration module of the purification device according to the invention; (1) ultrafiltration module, (2) column containing the IMAdSEC gel, (3) pumps, (4) ultrafiltrate,
- Figure 8 illustrates the capacity of the device illustrated in Figure 7 for the removal of β2-microglobulin from an ultrafiltrate of a uremic patient,
- Figure 9 illustrates the electrophoretic analysis of the fractions separated by chromatography illustrated in Figure 8; **1**: ultrafiltrate; **2**: 15 minutes of passage over the IMAdSEC gel; **3**: 30 minutes of passage over the IMAdSEC gel; **4**: 120 minutes of passage over the IMAdSEC gel; **5**: fraction eluted at pH 5.0; **6**: fraction eluted at pH 4.0; **7** fraction eluted at pH 3.0; **8**: fraction eluted with EDTA, **9**: protein standard,
- Figure 10 represents a hemodialysis system comprising the device according to the invention; (1) hemofilter, (2) hemodialyzer, (3) IMAdSEC column, (4) hemodialysis generator, (5) blood pump and (6) ultrafiltration pump.

EXAMPLE 1

Determination of the specificity and of the capacity of an IMAdSEC gel: (Novarose®-PEG/IDA-copper) for β2-microglobulin

1. <u>Synthesis of the Novarose®-PEG/IDA-copper gel</u>:

<u>Step 1</u>: coupling of PEG and creation of the cut-off of the gel:

10 g of Novarose® Act High 100/40 (INOVATA, Bromma, Sweden), previously dried by suction, are taken up in 5 ml of 1 M Na_2CO_3, pH > 12 and 5 ml of deionized water. 5 ml of 1 M Na_2CO_3, pH > 12, 5 ml of deionized

water and 30 ml of NH_2-PEG-NH_2 at 10% in 1 M Na_2CO_3, pH > 12, are added. The mixture is left under gentle stirring at room temperature (22°C) for 1 to 24 hours depending on the desired cut-off (this time is 4 hours for a cut-off of 20 kDa which is the desired cut-off for β2-microglobulin).

Step 2: coupling of the ligand : iminodiacetic acid (IDA).

The gel obtained in step 1 is rinsed on sintered material (by suction) with a solution of deionized water. It is resuspended in a solution comprising 15 ml of 1 M Na_2CO_3, pH > 12, 15 ml of deionized water, and 10 ml of a solution of IDA at 10% in 1 M Na_2CO_3, pH > 12. The mixture is left under gentle stirring at room temperature (22°C) for 48 hours. The IMAdSEC gel is rinsed on sintered material successively with deionized water, with a 1 M solution of sodium hydroxide, with deionized water, with a 0.1M solution of hydrochloric acid, and then with deionized water. The gel thus obtained is kept at 4°C in a solution of 20% ethanol until it is used.

Step 3: coupling of the metal ions (copper Cu II ions):

The metal load is prepared using an aqueous solution of copper sulfate at 50 mM under conventional conditions.

2. Preparation of the biological solutions

The products are derived from the hemofiltration of blood during an ultrafiltration session in the context of the treatment of uremic patients (Figure 2). Ultrafiltrates (pH 7.2, 13 mS/cm) are used whose β2-microglobulin concentration varies from 7 to 20 mg/l according to the patients.

3. Specificity of the Novarose®-PEG/IDA-copper gel for β2-microglobulin compared with gels without sieving Sépharose®4B-IDA-copper and Novarose®-IDA-copper

PROCEDURE:

3 gels were tested: Sépharose®4B-IDA-copper, Novarose®-IDA-copper (IMAC gels), and Novarose®-PEG/IDA-copper (IMAdSEC gel), for their capacity to adsorb the molecules of the ultrafiltrate from a uremic patient. The Sépharose® 4B-IDA gel was prepared according to the protocol described by Sundberg and Porath (J. Chromatogr., 1974, 90, 87-98). The Novarose®-IDA gel results from the same protocol as that described above at point 1 for the synthesis of the IMAdSEC gel, where only the second and the third steps were carried out (no prior activation of the gel with PEG). 2 ml of gel are applied to a column (diameter 1 cm) and low-pressure chromatography (1 ml/min) is carried out. 10 ml of ultrafiltrate from a patient, whose β2-microglobulin concentration is 20 µg/ml, are passed over each of the 3 different gels in closed circuit for 20 minutes. The equilibration and the rinsing of each column after adsorption of the ultrafiltrate are performed with an MMA buffer of pH 7.0 (MMA = MOPS, MES, Acetate, 25 mM each). The elution is carried out with a discontinuous decreasing pH gradient (buffer, 25 mM MMA, pH 6.0, then pH 5.0, then pH 4.0 and 25 mM glycine at pH 3.0), and then with a solution of EDTA (50 mM) to detach the copper. The protein content is measured during the chromatography by reading the optical density (λ = 280 nm) with a detector placed at the outlet of the column. The assay of β2-microglobulin is carried out by an immunological assay (rabbit polyclonal antibody anti-human β2-microglobulin, Dako, Denmark) using a nephelometry apparatus (Beckman, USA). The various fractions are analyzed by SDS-PAGE electrophoresis, according to the protocol described by Laemmli (Nature, 1970, 227, 680-685), and staining of the proteins with silver nitrate. After desalting and concentration, the fractions are analyzed by mass spectrometry (ESI-MS for "*ElectroSpray Ionisation Mass Spectrometry*" technique), whose sensitivity, determining the mass to the nearest dalton, makes it possible to identify the molecules.

RESULTS:

* The chromatography on Sépharose® 4B-IDA-copper gel (Figure 3a) shows that, while the β2-microglobulin has a high affinity for the chelated copper, its elution occurs in the same fractions as the albumin (Figure 4A). All the proteins of the ultrafiltrate are adsorbed onto the gel, which therefore exhibits no specificity for β2-microglobulin.

* The chromatography on Novarose®-IDA-copper gel (Figure 3B) also shows that this type of gel allows the adsorption of all the proteins of the ultrafiltrate (Figure 4B). Its capacity in relation to copper which is lower than that of Sépharose®-4B-IDA results, on the other hand, in elutions of proteins during the discontinuous pH gradient, unlike the Sépharose® 4B-IDA gel (Figure 4B versus 4A). Like the latter, it does not offer specificity for β2-microglobulin (Figure 4B).

* The chromatography on Novarose®-PEG/IDA-copper gel, on the other hand, allowed the adsorption of solely the β2-microglobulin of the ultrafiltrate from the patient. Its elution takes place at pH 3.0 as two distinct peaks (Figure 4C).

In the three types of chromatography, the analyses by nephelometry confirm the complete disappearance of β2-microglobulin from the ultrafiltrate fraction passed over the 3 types of gel and its elution from the column.

ESI-MS analysis shows that the chromatography on IMAdSEC gel makes it possible to pass from a fraction consisting of a starting mixture: albumin + β2-microglobulin, to a fraction eluted at pH 3.0 which contains only β2-microglobulin (Figure 5A versus 5B).

These results show the affinity of β2-microglobulin for the ligand (chelated metal, here copper) and the specificity offered by the molecular sieving (coupling of PEG) of the IMAdSEC gel compared with the conventional IMAC gels.

4. <u>Capacity of the IMAdSEC-copper gel for β2-microglobulin</u>

PROCEDURE:

50 ml of ultrafiltrate from a uremic patient, containing 350 µg of β2-microglobulin (that is a β2-microglobulin concentration of 7 µg/ml) circulates in closed circuit for 150 minutes on 0.65 ml of IMAdSEC gel under the same chromatographic conditions as above (flow rate = 1 ml/min). The elution is carried out directly at pH 4.0 (Figure 6).

RESULTS:

After 150 minutes, the β2-microglobulin concentration measured by nephelometry is 2.3 µg/ml, that is a remaining β2-microglobulin quantity of 115 µg. Consequently, 235 µg of β2-microglobulin were bound to the 0.65 ml of gel, which corresponds to a binding capacity of the IMAdSEC-copper gel of 360 µg/ml. SDS-PAGE and ESI-MS analysis of the fractions was carried out as described above. The quantity of β2-microglobulin, eluted at pH 4.0, is about 180 µg instead of 235 µg expected. The difference may be explained by the absence of measurement of the rinsing and EDTA fractions which are also likely to contain β2-microglobulin.

These results suggest that, taking into account these performances and this specificity for β2-microglobulin, a column of 500 to 750 ml of IMAdSEC-copper gel would make it possible to remove 250 mg of β2-microglobulin, a quantity which corresponds to 5 liters of blood at a β2-microglobulin concentration of 50 mg/l.

EXAMPLE 2

Separation and purification of β2-microglobulin by a device comprising the coupling of an ultrafiltration module and an IMAdSEC column

PROCEDURE

The assembly represented in Figure 7 is used. The ultrafiltration module (1) used is composed of 100 Polysulfone hollow fibers drawn from a commercial ultrafiltration module model Fresenius F80.

50 ml of ultrafiltrate from a uremic patient (β2-microglobulin concentration = 7 µg/ml) are passed in a closed circuit for 3 hours on the ultrafiltration/column of IMAdSEC gel (0.65 ml of IMAdSEC gel) minimodule assembly. The chromatography conditions are those of Example 1, namely: buffer, 25 mM MMA, pH 6.0, then pH 5.0, then pH 4.0 and 25 mM glycine at pH 3.0, then 50 mM EDTA to elute the copper chelated on the gel.

After 3 hours, the β2-microglobulin concentration in the reservoir is measured by nephelometry.

RESULTS

The concentration passes from 7 µg/ml of β2-microglobulin (that is a starting quantity of 350 µg) to about 1 µg/ml (50 µg of β2-microglobulin remaining). Consequently, about 300 µg of β2-microglobulin were bound to the 0.65 ml of IMAdSEC gel, which corresponds to a binding capacity of the IMAdSEC gel for β2-microglobulin of 461 µg/ml.

ESI-MS (Figure 8) and SDS-PAGE (Figure 9) analysis of the fractions show that the β2-microglobulin was adsorbed specifically by the IMAdSEC gel. It is eluted as two main fractions at pH 4.0 and pH 5.0.

These results suggest that the IMAdSEC gel could be useful for the separation of biomolecules and their isoforms such as for example normal β2-microglobulin and glycated β2-microglobulin.

AMENDED CLAIMS

[received by the International Bureau on 10 April 2000 (10.04.00); original claims 1-16 replaced by new claims 1-9 (2 pages)]

1. Device for removing biomolecules comprising an ultrafiltration module optionally upstream and in series with a dialysis module, characterized in that this device further comprises a column containing an adsorbent gel combining the properties of size exclusion and affinity chromatographies, said adsorbent gel consisting essentially of a polysaccharide matrix onto which is grafted a polymer coupled to an affinity ligand and having an adjustable cut-off of between 2 kDA and 60 kDa, said column being mounted branching from said ultrafiltration module.

2. Device according to claim 1, characterized in that the adsorbent gel consists of a matrix based on an agarose derivative onto which is grafted polyethylene glycol coupled to iminodiacetic acid itself coupled to copper(I) ions and having a cut-off of 20 kDa.

3. Device for separating and purifying biomolecules comprising a column containing an adsorbent gel combining the properties of size exclusion and affinity chromatographies, said gel consisting essentially of a polysaccharide matrix onto which is grafted a polymer coupled to an affinity ligand and having an adjustable cut-off of between 2 kDa and 60 kDa, said column being optionally mounted branching from a filtration module.

4. Device according to claim 3, characterized in that the adsorbent gel consists of a matrix based on an agarose derivative onto which is grafted polyethylene glycol coupled to iminodiacetic acid itself coupled to copper(I) ions and having a cut-off of 20 kDa.

5. Device according to claim 2 or claim 4, characterized in that the biomolecule is serum β2-microglobulin.

6. Use of the device according to claims 1 to 5 for removing biomolecules from blood, with the exception of extracorporeal dialysis.

7. Use according to claim 6, characterized in that the device comprises an adsorbent gel consisting of a matrix based on an agarose derivative onto which is grafted polyethylene glycol coupled to iminodiacetic acid itself coupled to copper(I) ions and having a cut-off of 20 kDa.

8. Use according to claim 7, characterized in that the biomolecule is serum β2-microglobulin.

9. Device according to any one of claims 1 to 5, characterized in that the device is an extracorporeal dialysis system.

Abstract

The invention concerns the use of an adsorptive size exclusion chromatography gel, said gel essentially consisting of a polysaccharide matrix whereon is grafted a polymer coupled with an affinity ligand and having a cleavage threshold ranging between 2 kDa and 60 kDa for eliminating a purifying biomolecules.

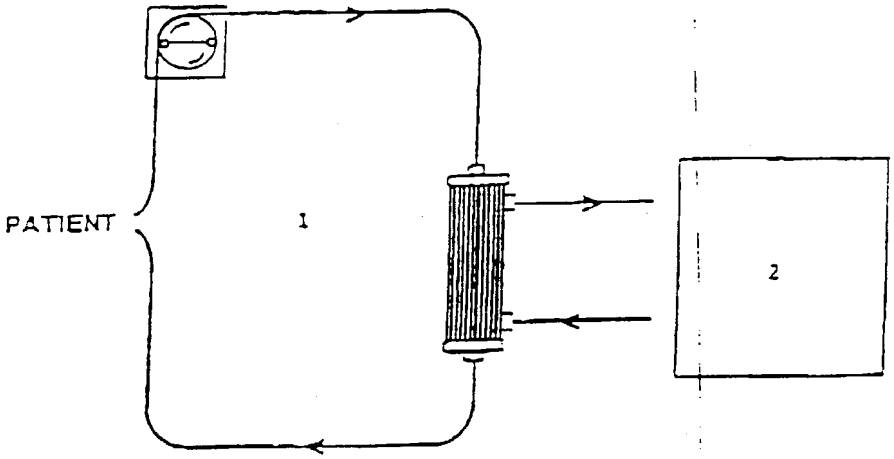

Figure 1

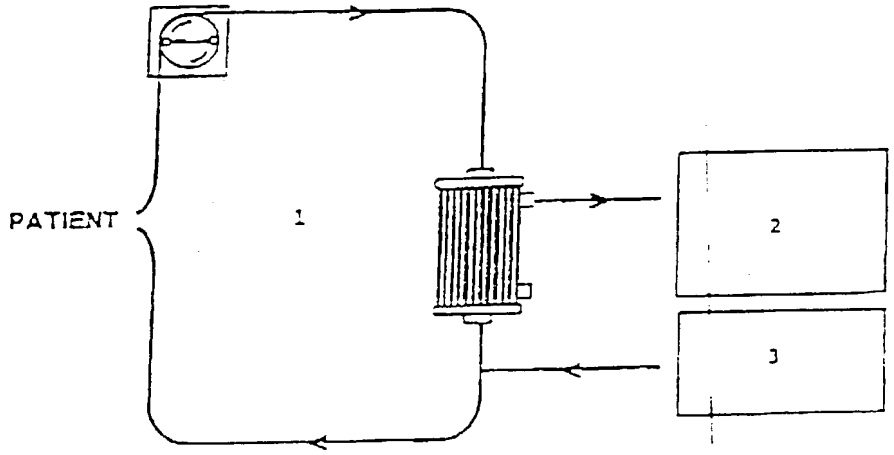

Figure 2

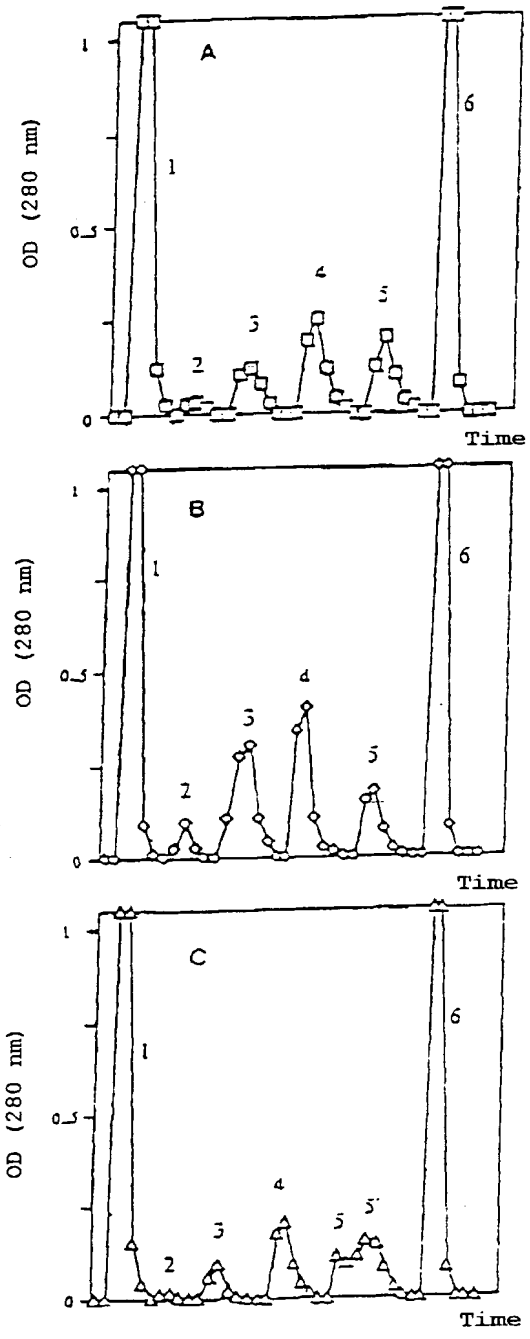

Figure 1

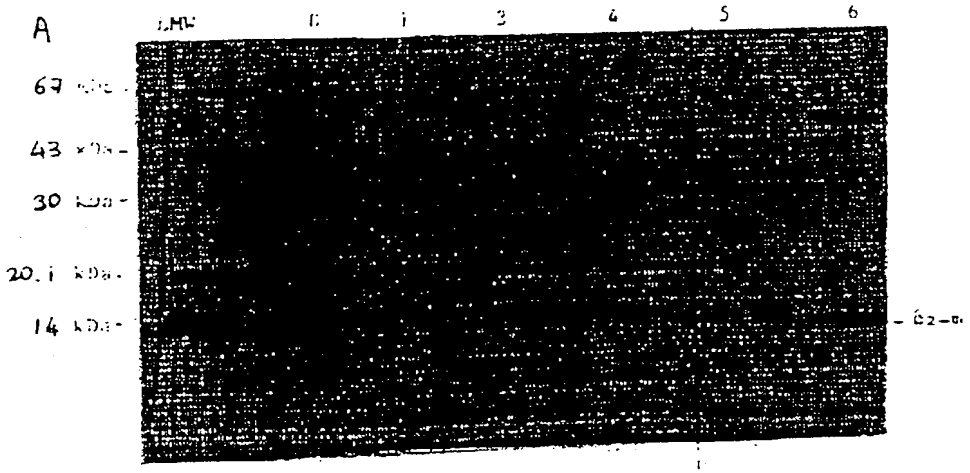

Figure 4a

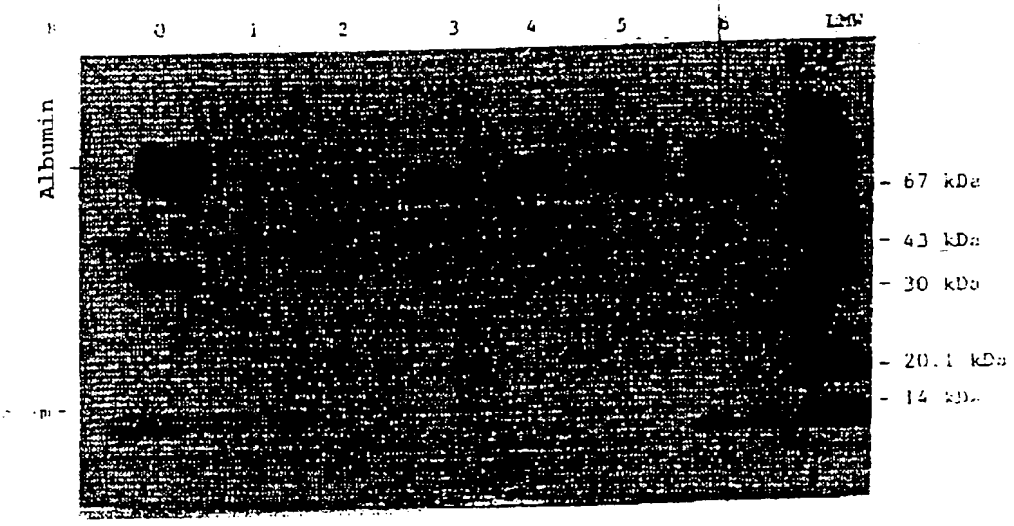

Figure 4b

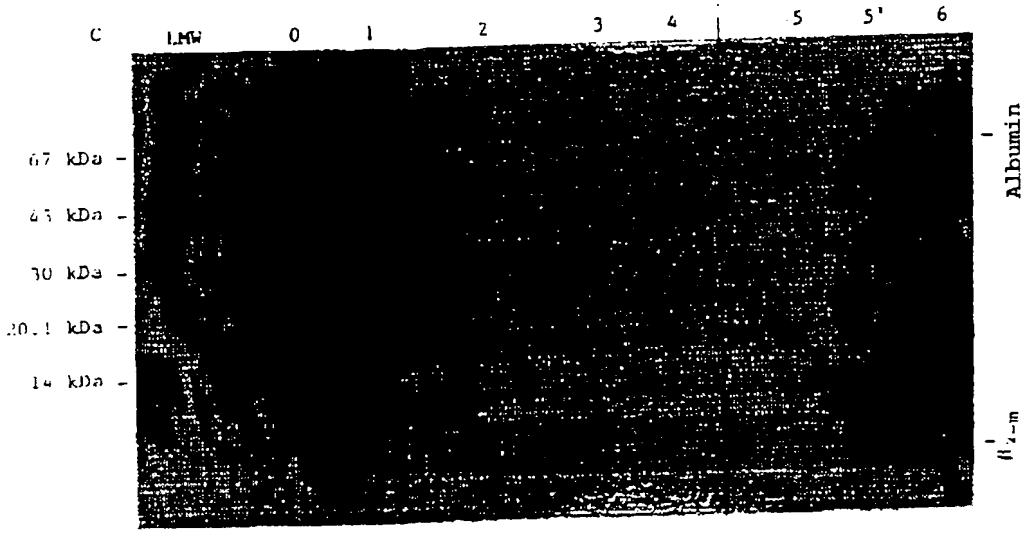

Figure 4c

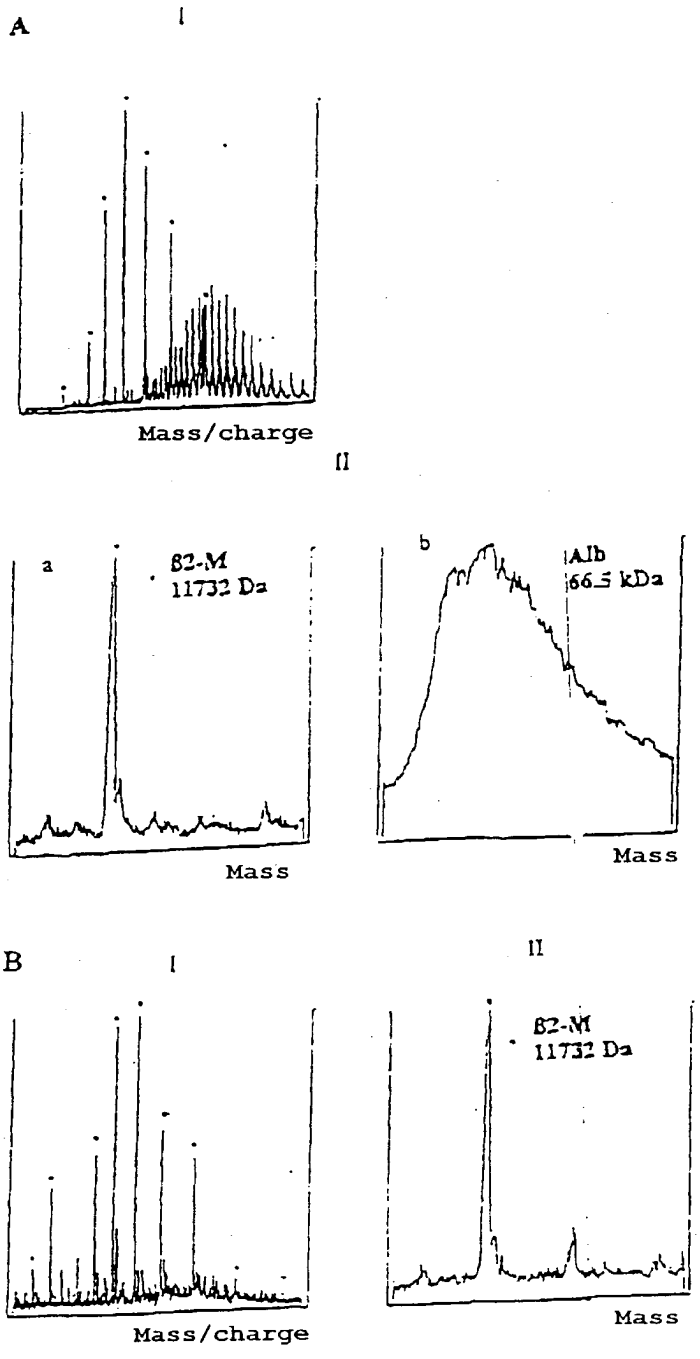
A
I
Mass/charge
II
a
β2-M
11732 Da
Mass
b
Alb
66.5 kDa
Mass
B
I
Mass/charge
II
β2-M
11732 Da
Mass

Figure 5

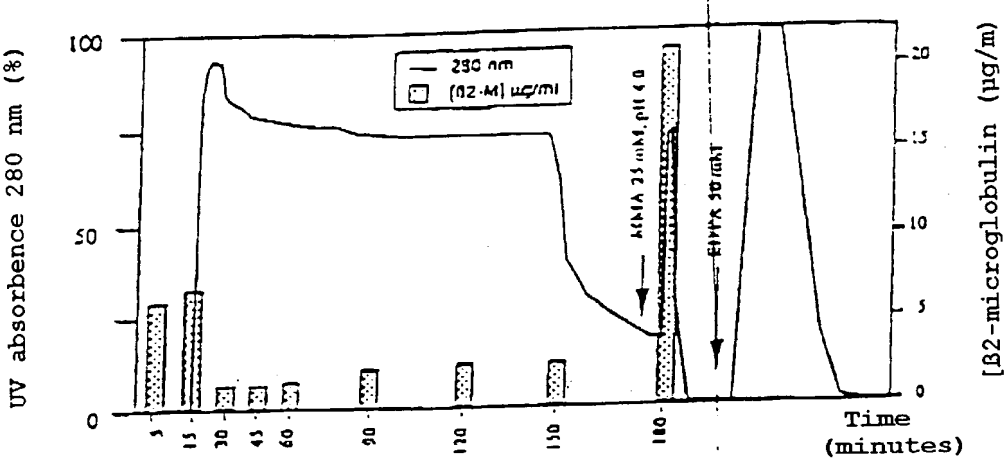

Figure 6

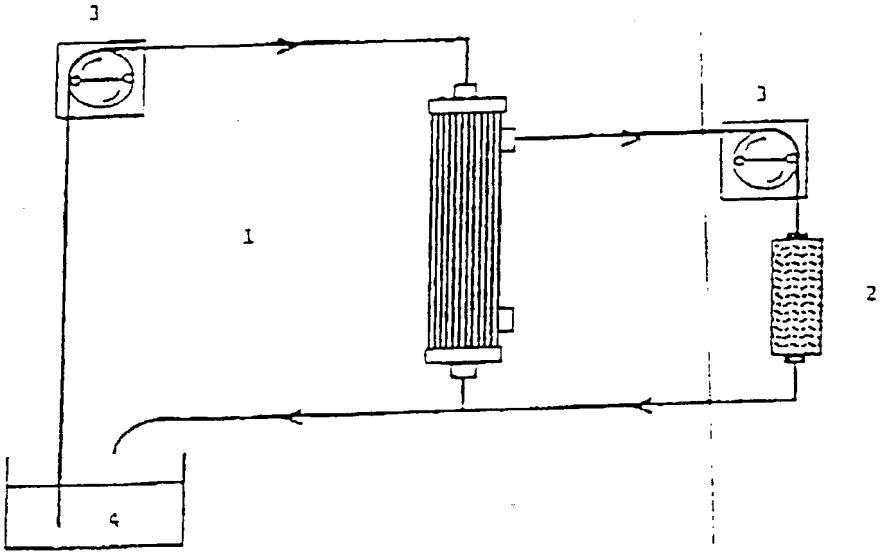

Figure 7

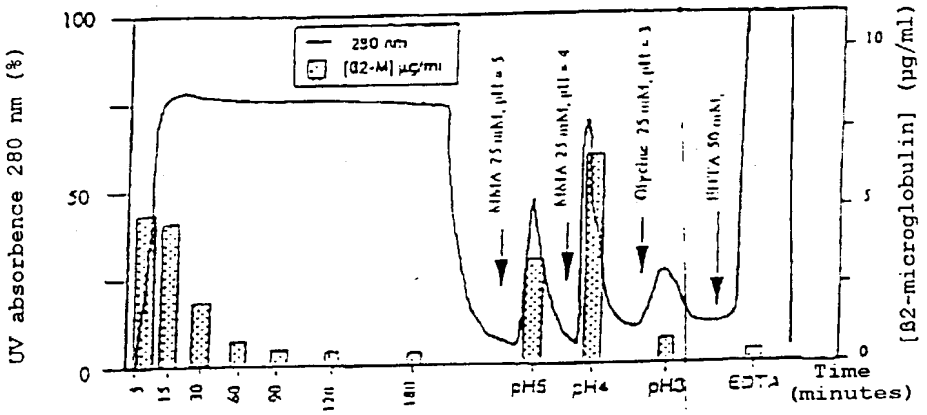

Figure 8

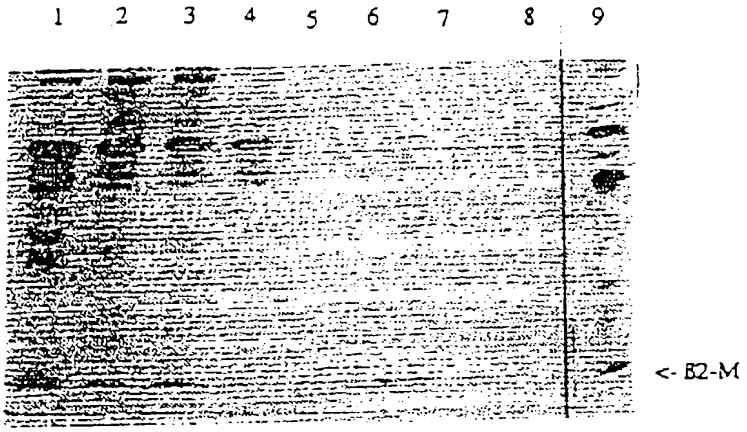

Figure 9

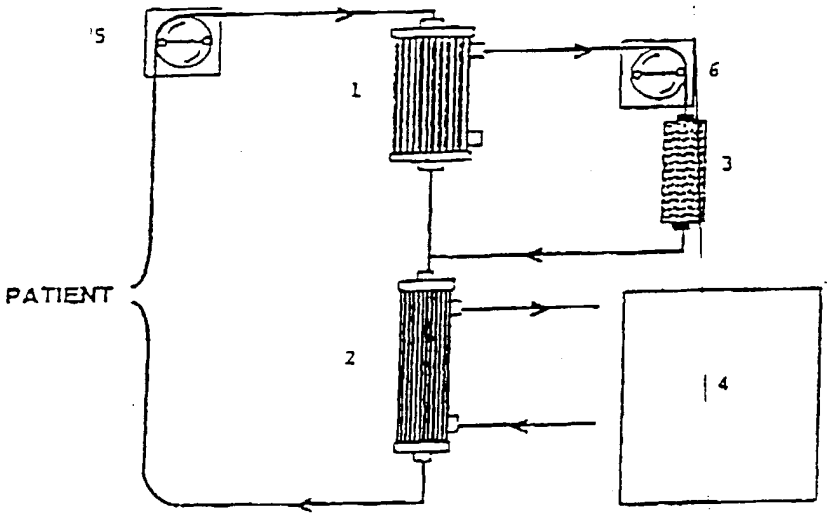

Figure 10

N/Ref. 61443 N/Ref : SGdL1644/38 US DOCUMENT A #B

Declaration and Power of Attorney for Patent Application
Déclaration et Pouvoirs pour Demande de Brevet
French Language Declaration

En tant l'inventeur nommé ci-après, je déclare par le présent acte que :

Mon domicile, mon adresse postale et ma nationalité sont ceux figurant ci-dessous à côté de mon nom.

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) de l'objet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée

et dont la description est fournie ci-joint à moins

☐ ci-joint

☐ a été déposée le

sous le numéro de demande des Etats-Unis ou le numéro de demande international PCT

et modifiée le

(le cas échéant).

Je déclare par le présent acte avoir passé en revue et compris le contenu de la description ci-dessus, revendications comprises, telles que modifiées par toute modification dont il aura été fait références ci-dessus.

Je reconnais devoir divulguer toute information pertinente à la brevetabilité, comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations.

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed an for which a patent is sought on the invention entitled

Use of an adsorbent gel for eliminating and purifying biomolecules

the specification of which :

☐ is attached hereto.

☐ was filed on

as United States Application Number or PCT International Application Number.
PCT/FR99/02635 filed on October 28, 1999

and was amended on

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations,§ 1.56.

French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119(a)-(d) ou § 365(b) du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur ou, en vertu du Titre 35, § 365(a) du même Code, sur toute demande internationale PCT désignant au moins un pays autre que les Etats-Unis et figurant ci-dessous et, en cochant la case, j'ai aussi indiqué ci-dessous toute demande étrangère de brevet, tout certificat d'inventeur ou toute demande internationale PCT ayant date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

I hereby claim foreign priority under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below, and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign application(s)
Demande(s) de brevet antérieure(s) dans un autre pays.

(Number) (Numéro)	(Country) (Pays)	(Day/Month/Year Filed) (Jour/Mois/Anné de dépôt)	Priority claimed Droit de priorité revendiqué ☒ Yes Oui	☐ No Non
98/13655	FRANCE	30/10/1998		
(Number) (Numéro)	(Country) (Pays)	(Day/Month/Year Filed) (Jour/Mois/Anné de dépôt)	☐ Yes Oui	☐ No Non

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 119(e) du Code des Etats-Unis, de toute demande de brevet provisoire effectuée aux Etats-Unis et figurant ci-dessous.

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis, ou en vertu du Titre 35, § 365© du même Code, de toute demande internationale PCT désignant les Etats-Unis et figurant ci-dessous et, dans la mesure où l'objet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande antérieure américaine ou internationale PCT, en vertu des dispositions du premier paragraphe du Titre 35, § 112 du code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la brevetabilité, comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la demande antérieure et la date de dépôt de la demande nationale ou internationale PCT de la présente demande :

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)	(Status) (patented, pending, abandoned) (Statut) (breveté, en cours d'examen, abandonné)
(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)	(Status) (patented, pending, abandoned) (Statut) (breveté, en cours d'examen, abandonné)

Je déclare que par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique ;et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la section 1001 du Titre 18 du Code de Etats-Unis, et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hebery declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocats(s) et/ou agent(s) suivant(s) pour qu'ils poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire s'y rapportant avec l'Office des brevets et des marquees : (mentionner le nom et le numéro d'enregistrement).

POWER OF ATTORNEY : As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to persecute this application and transact all bussiness in the Patent and Trademark Office connected therewith : (list name and registration number)

29

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(703) 413-3000

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Signature de l'inventeur Date M.A.V. 10·05·2001	Inventor's signature Date
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Signature de l'inventeur Date	Second inventor's signature Date
Domicile 02200 SOISSONS (France)	Residence
Nationalité Française	Citizenship
Adresse Postale 5, Rue de l'Intendance 02200 SOISSONS (France)	Post Office Address

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Suppply similar information and signature for third and subsequent joint inventors.)

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocats(s) et/ou agent(s) suivant(s) pour qu'ils poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire s'y rapportant avec l'Office des brevets et des marques : (mentionner le nom et le numéro d'enregistrement).

POWER OF ATTORNEY : As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to persecute this application and transact all bussiness in the Patent and Trademark Office connected therewith : (list name and registration number)

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Nationalité Française	Citizenship
Adresse Postale 5 Square Charles Gounot 60200 COMPIEGNE (France)	Post Office Address
Nom complete du second co-inventeur, le cas echeant **PITIOT Olivier**	Full name of second joint inventor, if any
Signature de l'inventeur Date 09 Mai 2001	Second inventor's signature Date
Domicile ~~02200 SOISSONS~~ (France) 69290 SAINT-GENIS-LES-OLLIÈRES	Residence FRX
Nationalité Française	Citizenship
Adresse Postale ~~5, Rue de l'Intendance 02200 SOISSONS~~ (France)	Post Office Address
3. ALLEE DE VAL FONTAINE 69290 ST-GENIS-LES-OLLIÈRES	

2-00

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Suppply similar information and signature for third and subsequent joint inventors.)

French Language Declaration

3-00

Nom complete du troisième co-inventeur, le cas échéant **LEGALLAIS Cécile**	Full name of third joint inventor, if any
Signature de l'inventeur Date 09/05/01	Third inventor's signature Date
Domicile 60340 VILLERS SOUS SAINT LEU (France)	Residence FRX
Nationalité Française	Citizenship
Adresse Postale 15, Rue du Château d'eau 60340 VILLERS SOUS SAINT LEU (France)	Post Office Address
Nom complete du quatrième co-inventeur, le cas echeant **MORINIERE Philippe**	Full name of fourth joint inventor, if any
Signature de l'inventeur Date	Fourth inventor's signature Date
Domicile 80000 AMIENS (France)	Residence
Nationalité Française	Citizenship
Adresse Postale 102 Avenue Foy 80000 AMIENS (France)	Post Office Address
Nom complete du cinquième co-inventeur, le cas echeant	Full name of fifth joint inventor, if any
Signature de l'inventeur Date	Fifth inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse Postale	Post Office Address
Nom complete du sixième co-inventeur, le cas echeant	Full name of sixth joint inventor, if any
Signature de l'inventeur Date	Sixth inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse Postale	Post Office Address

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Supply similar information and signature for third and subsequent joint inventors.)

French Language Declaration

Nom complete du troisième co-inventeur, le cas échéant **LEGALLAIS Cécile** Signature de l'inventeur Date 09/05/01	Full name of third joint inventor, if any Third inventor's signature Date
Domicile 60340 VILLERS SOUS SAINT LEU (France)	Residence
Nationalité Française	Citizenship
Adresse Postale 15, Rue du Château d'eau 60340 VILLERS SOUS SAINT LEU (France)	Post Office Address
Nom complete du quatrième co-inventeur, le cas echeant **MORINIERE Philippe** Signature de l'inventeur Date 3/5/01	Full name of fourth joint inventor, if any Fourth inventor's signature Date
Domicile 80000 AMIENS (France)	Residence FRX
Nationalité Française	Citizenship
Adresse Postale 102 Avenue Foy 80000 AMIENS (France)	Post Office Address
Nom complete du cinquième co-inventeur, le cas echeant Signature de l'inventeur Date	Full name of fifth joint inventor, if any Fifth inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse Postale	Post Office Address
Nom complete du sixième co-inventeur, le cas echeant Signature de l'inventeur Date	Full name of sixth joint inventor, if any Sixth inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse Postale	Post Office Address

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Supply similar information and signature for third and subsequent joint inventors.)

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